

Activation of Chemicals into Mutagens by Green Plants: A Preliminary Discussion

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This paper is a review of recent studies that demonstrate the activation of chemicals (especially pesticides) into mutagens by green plants. Such activation of pesticides may be hazardous to the public health because of their widespread use in agriculture and the current lack of information that exists about such processes. The mutagenic properties of the *s*-triazine herbicides (atrazine, simazine, and cyanazine) as exhibited in various assay systems are discussed. *In vivo*, *in vitro*, and *in situ* plant assays are presented, and the maize *wx* locus assay is discussed.

Introduction

The activation of chemicals into mutagens by green plants is a recently discovered hazard. The discovery of plant activation of pesticides into mutagens (1-5) brings into question the effects of such agents upon the public health. The current procedures used to register pesticides do not consider the question of their activation into genotoxins. It is unknown whether such mutagens can enter the food chain via the produce or water supplies.

In Vivo, *In Vitro* and *In Situ* Assays

Three techniques involved in research on plant activation are the *in vivo*, *in vitro*, and *in situ* methods. The *in vivo* method involves growing plants exposed to a suspect chemical, homogenizing the plant tissues, and making an extract from the treated plants. Control plants are not treated with the chemical under study and an extract is made. Microbial indicator organisms are exposed to the extracts and the mutagenic or recombinogenic effects of the extracts obtained from the treated plants are compared with their controls. This method has advantages in that it mimics the conditions encountered in the field and it can detect mutagenic

agents that accumulate in the sporophyte or grain (6) (Table 1). The *in vitro* method involves incubating a test chemical with an untreated plant homogenate supplemented with cofactors. The homogenate is assayed by microbial indicator organisms to detect the presence of newly synthesized mutagens (Table 1). This method has the advantages of being very similar to the *Salmonella*/mammalian-microsome activation assay (7), and the kinetics of the activation process can be monitored. In the studies cited in Table 1 the chemicals under investigation were tested directly with microbial assays and were generally found not to be mutagens. The genetic endpoint of the *in situ* method is reversion at the *waxy* (*wx*) locus of *Zea mays* microgametophytes (pollen grains). With this procedure chemicals, especially pesticides, can be evaluated for their mutagenic properties under "real world" conditions. While this method does not have the controlled laboratory conditions of the *in vivo* and *in vitro* methods, it does detect the presence of a mutagen within the ecological dynamics of the agricultural field.

s-Triazine Herbicides

The *s*-triazine herbicides are widely used pesticides in the United States. These compounds have been chosen for studies in plant activation because of their widespread use in agriculture and the contradictory results concerning their mutagenic properties.

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Table 1. Studies on plant activation of chemicals into mutagens conducted at various laboratories.

Agent	Plant tissue	Assay type	Microbial indicator	Response ^a	Reference
Atrazine	<i>Z. mays</i>	<i>In vivo</i>	<i>S. cerevisiae</i> , gene reversion and conversion	+	1-3
Atrazine	<i>Z. mays</i>	<i>In vivo</i>	<i>E. coli</i> , lethality of repair deficient strains	+	<i>b</i>
Atrazine	<i>Z. mays</i>	<i>In vivo</i>	<i>E. coli</i> , forward mutation to streptomycin resistance	+	<i>b</i>
Atrazine	<i>Z. mays</i>	<i>In vivo</i>	<i>S. typhimurium</i> , TA98 TA100	- +	<i>c</i> <i>c</i>
Atrazine	<i>Z. mays</i>	<i>In vivo</i>	<i>S. cerevisiae</i> , gene conversion	-	<i>d</i>
Cyanazine	<i>Z. mays</i>	<i>In vivo</i>	<i>S. typhimurium</i> , TA98 TA100	- +	<i>c</i> <i>c</i>
Cyanazine	<i>Z. mays</i>	<i>In vivo</i> , <i>In vitro</i>	<i>S. typhimurium</i> , TA98 TA100	- +	<i>e</i> <i>e</i>
Alachlor	<i>Z. mays</i>	<i>In vivo</i>	<i>S. cerevisiae</i> , gene conversion	±	4
Propachlor	<i>Z. mays</i>	<i>In vivo</i>	<i>S. cerevisiae</i> , gene conversion	±	4
Heptachlor	<i>Z. mays</i>	<i>In vivo</i>	<i>S. typhimurium</i> , TA98 TA100	- -	<i>f</i> <i>f</i>
Chlordane	<i>Z. mays</i>	<i>In vivo</i>	<i>S. typhimurium</i> , TA98 TA100	- -	<i>f</i> <i>f</i>
1,2-dibromoethane	<i>T. reflexa</i>	<i>In vitro</i>	<i>E. coli</i> , reversion <i>A. nidulans</i> , reversion	^g ^g	5 5

^a Response: +, positive response; ± weak positive response; - negative response.

^b G. Warren and S. J. Rogers, Montana State University, unpublished data.

^c S. J. Rogers, Montana State University, unpublished data.

^d D. D. Sumner, Ciba-Geigy Corporation, unpublished data.

^e J. M. Gentile, Hope College, unpublished data.

^f J. M. Gentile and M. J. Plewa, unpublished data.

^g 1,2-Dibromoethane is a direct acting mutagen, however, the *Tradescantia* homogenate caused an enhancement of the mutagenic potency of the agent.

The results of cytogenetic studies on *s*-triazine herbicides and related compounds indicate that these agents induce both mitotic and meiotic damage. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) and simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine] increased the frequency of mitotic chromosome aberrations in root-tip cells of barley (*Hordeum vulgare*) (8-10) and *Vicia faba* (11). In natural vegetation damage to mitotic chromosomes induced by various herbicides including simazine has recently been reported (12). Another widely distributed *s*-triazine herbicide, cyanazine, {2-[(chloro-6-(ethylamino)-*s*-triazine-2-yl)amino]-2-methyl-propionitrile} caused chromosomal aberrations in root-tip cells of *Tradescantia reflexa* and *V. faba* (13). Atrazine and simazine induced chromosome aberrations in meiotic cells of barley (14) and atrazine induced meiotic chromosome damage in microsporocytes of *Sorghum vulgare* (15, 16). The herbicides atrazine and 2,4-D (2,4-dichlorophenoxy-

acetic acid) each induced damage to meiotic chromosomes of grain sorghum. When the plants were treated simultaneously with both herbicides the frequency of chromosomal aberrations increased due to a synergistic effect (17). The practice of combining pesticides to increase the range of pest control is a common procedure in modern agriculture. Lee et al. reported meiotic chromosome aberrations in *S. vulgare* plants treated with atrazine. The frequency of chromosomal aberrations was higher in F₁ plants than in the parental plants originally exposed or in F₂ plants (18). Finally, agricultural workers who were employed as custom applicators of herbicides, including atrazine, had a maximum of a four-fold increase in chromosome aberrations detected in their blood lymphocyte cultures as compared to a control population (19). At variance with the above conclusions, one cytological study reported that simazine did not induce mitotic instabilities at nontoxic concentrations in *T. reflexa* or *V. faba* (20) and in another report

atrazine was found not to induce cytogenetic damage in *V. faba*, *H. vulgare*, and *S. vulgare* (21).

Controversy exists whether certain radiolabeled *s*-triazines can be incorporated into the nucleic acids of *Escherichia coli*. The herbicide prometryne [2,4-bis(isopropylamino)-6-methylmercapto-*s*-triazine] and cyanuric acid were reported to partially replace uracil in RNA and thymine in DNA (22). Seiler observed a small amount of incorporation of radiolabeled 2-hydroxy-4-amino-6-ethylamino-*s*-triazine into *E. coli* DNA (J. P. Seiler, personal communication, Swiss Federal Research Station for Fruit Growing, Viticulture and Horticulture, Wädenswil, Switzerland.) However, another study reported no incorporation of ring-¹⁴C-labeled *s*-triazines in *E. coli* nucleic acids (23).

Of interest is the finding that atrazine enhanced the synthesis of chromatin-directed RNA in soybean (*Glycine max*) seedlings. This stimulation of chromatin-directed RNA synthesis was not observed in maize seedlings or when 2-hydroxy-4-(ethylamino)-6-isopropylamino-*s*-triazine was substituted for atrazine (24).

Many *s*-triazine compounds have been evaluated for their ability to induce point mutations. Direct microbial assays have demonstrated that atrazine and simazine do not induce point mutations (25–27), although very weak mutagenic responses of 2-hydroxy-4,6-bis(ethylamino)-*s*-triazine and 2-chloro-4,6-diamino-*s*-triazine in two strains of *Salmonella typhimurium* were observed (26). Atrazine, simazine, and other *s*-triazines did not induce gene conversion in *Saccharomyces cerevisiae* (28). However, atrazine and simazine induced recessive mutations in F₂ plants of *H. vulgare* (8). Atrazine, simazine, and cyanazine induced dominant lethals in *Drosophila melanogaster* (29–31). Atrazine and simazine induced sex-linked recessive lethal mutations when the herbicides were injected intraabdominally into male *Drosophila* (31). When administered by larval feeding, atrazine induced sex-linked recessive lethal mutations and significantly increased the rate of X or Y chromosome loss (30, 31). Cyanazine induced a higher rate of sex-linked recessive lethals, however, this increase was not significant (31). We reported that atrazine was activated *in vivo* by maize into an agent(s) that induced reversion and gene conversion in *S. cerevisiae* (2, 3). This observation of plant activation of atrazine into a mutagen was independently verified by G. Warren and S. J. Rogers (personal communication, Montana State University, Bozeman, Montana). In their study they observed that extracts from maize sporophytes treated with atrazine contained a DNA damaging material. Repair deficient strains of *E. coli* B were analyzed for lethality and strains *uvrA*

and *recA* reacted to the treated maize extracts. Also such plant extracts induced mutations to streptomycin resistance in both wild-type and *uvrB E. coli*. Extracts from untreated plants did not induce lethality or mutation in the *E. coli* assays. In a study employing the *S. typhimurium* strains TA98 and TA100, maize extracts from plants treated with atrazine or cyanazine induced a significantly increased reversion frequency in strain TA100. The mutagenic agent(s) in the maize extracts were uninfluenced by the presence or absence of mammalian liver microsome preparations. Extracts from untreated control plants did not induce mutations in strains TA98 and TA100 (S. J. Rogers, personal communication, Montana State University, Bozeman, MT). However, a communication by D. Sumner of Ciba-Geigy Corporation indicated that plant activation of atrazine was not observed using *S. cerevisiae* as the microbial indicator organism. *In situ* studies using the maize *waxy* locus reversion assay indicated atrazine, simazine and cyanazine induced reversion at the *wx* locus in pollen grains (32, 33) (Table 2).

Thus, the majority of data reported in the investigations outlined above indicate that the *s*-triazine herbicides atrazine, simazine and cyanazine induce both mitotic and meiotic chromosome aberrations and are biologically activated into agents that induce point mutations.

The *waxy* Locus of *Z. mays*

Near the beginning of this century an altered endosperm trait in maize was introduced from China that was distinctly different from the American varieties of floury, sweet, flint, or pop. This novel variety was named “waxy” because the endosperm had the appearance of hard wax (34). Genetic studies on waxy maize confirmed that the *waxy* allele is recessive to starchy (*Wx*) and *wx* segregated in the F₂ generation as a Mendelian monohybrid (35). In waxy kernels, the starch of the endosperm contains only amylopectin, while in kernels carrying the dominant allele, *Wx*, the endosperms contain starch composed of a mixture of amylopectin and amylose (36, 37). Because of the presence of amylose, the endosperms of kernels carrying the *Wx* allele stain a dark blue-black color when reacted with iodine. When an iodine solution is reacted with endosperms of *wx/wx* kernels however, a red color is produced.

The enzymatic differences inherent in the endosperms of homozygous *wx* maize kernels as compared to those kernels carrying the dominant *Wx* allele were investigated by Nelson and Rines (38). Homozygous *wx* kernels contain the same amount

Table 2. Maize *wx* locus reversion assay of pesticides or combination of pesticides.

Pesticide	Active agent	Application rate of active agent, kg/ha	Number of pollen grains analyzed $\times 10^5$	Reversion frequency $\times 10^{-5}$
Control	—	—	25.89	5.56 \pm 0.98
Cyanazine	2-(4-chloro-6-ethylamino-s-triazine-2-yl amino)-2-methyl-propionitrile	2.86	4.71	28.23 \pm 3.36
Procyazine	2[[4-chloro-6-(cyclopropylamino)-s-triazine-2-yl]amino]-2-methyl propanenitrile	2.86	2.41	4.65 \pm 1.76
Control	—	—	19.91	4.16 \pm 1.10
Heptachlor	1,4,5,6,7,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoidene	0.22	7.30	10.87 \pm 3.06
Chlordane	1,2,4,5,6,7,8,8-octachlor-2,3,3a,4,7,7a-hexahydro-4,7-methanoindane	0.75	8.55	13.69 \pm 1.10
Control	—	—	9.75	3.19 \pm 0.67
Eradicane	S-ethyl dipropylthiocarbamate	7.20	8.09	5.31 \pm 1.64
Metolachlor	2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide	6.00	9.59	4.32 \pm 1.14
Atrazine	2-chloro-4-ethylamino-6-isopropylamino-s-triazine	3.07	9.50	8.92 \pm 1.29
Simazine	2-chloro-4,6-bis(ethylamino)-s-triazine	3.07	8.19	12.00 \pm 2.31
Eradicane + Cyanazine		3.60 + 2.59	8.83	9.18 \pm 1.46
Metolachlor + Cyanazine		2.24 + 1.79	5.17	11.08 \pm 3.11
Eradicane + Atrazine		3.60 + 1.54	8.86	10.77 \pm 1.59
Metolachlor + Atrazine		3.00 + 1.79	7.19	12.27 \pm 2.34

of starch as starchy kernels. However, the waxy kernels do not have the uridine-diphosphate-glucose transferase system. Since the starch in waxy kernels is entirely composed of amylopectin (36), this carbohydrate must be synthesized by a different biochemical pathway than the branching of amylose formed via uridine-diphosphate-glucose transferase (38). The use of the iodine test provided an early, rapid and accurate chemical assay for a genetic characteristic.

It was soon discovered that the *wx* phenotype could be detected by the iodine test in the microgametophytes (pollen grains). Pollen grains are functional haploids and in a heterozygous plant both alleles segregate according to Mendel's first law (39, 40). Furthermore, the data indicate that a single gene and its alleles can be similarly expressed in both the sporophytic and gametophytic generations.

Since the *wx* allele can be detected in single pollen grains it was suggested that this system be used in the study of the genetic fine structure of a locus in a higher eukaryote (41). These suggestions were based on the then recent discoveries that redefined the classical structure of the gene (42). The increase in genetic resolution, however, required very large populations (43) and a population in excess of 10^5 is usually impossible to analyze in higher eukaryotes. Maize pollen grains were suited to the problem of population size because great numbers could be analyzed rapidly. Also, at that time a few independently occurring *wx* mutations (heteroalleles) were collected and were available for *cis-trans* tests (44).

The starch type of a pollen grain is controlled by the genetic constitution of that pollen grain, not by the parental sporophyte. Thus, a genetic reversion of *wx* to *Wx* can be detected by scoring for pollen grains from plants that are homozygous *wx* and that stain a dark blue-black color when subjected to an iodine test (44, 45). Intragenic recombination between two different mutations at the *wx* locus can be analyzed by scoring for *Wx* pollen grains from plants that are intercrosses of lines representing the different heteroalleles (44, 45).

To date, 31 different heteroalleles have been mapped at the *wx* locus (46). Five of these mutations are controlling element alleles. These heteroalleles are of interest because they possess potentially functional *Wx* alleles that are prevented from functioning by the controlling element (47). Finally, the phenomenon of gene conversion (non-reciprocal intragenic recombination) has been reported at the *wx* locus (48).

Studies on forward mutation at the *wx* locus have demonstrated that acute and chronic γ -radiation induces a linear increase in mutation frequency with an increased dose of radiation (49). These data agree with studies on the induction of forward and reverse mutations at the *wx* locus of barley by ^{90}Sr incorporated in the soil (50). The effect of x-radiation on reversion frequencies of three *wx* heteroalleles, *wx-H21*, *wx-C*, and *wx-90*, and on the frequency of intragenic recombination among three different heteroallelic combinations has been reported (51). The authors found an increase in the

reversion frequency at the *wx-90* heteroallele and a significant decrease ($\alpha = 0.001$) in intragenic recombination between *wx-C* and *wx-90* heteroalleles.

We have conducted research using the maize *wx-C* heteroallele as a marker for a reversion assay for the detection of mutagenic properties of pesticides (32, 33). Data are presented in Table 2 for some of the pesticides and combination of pesticides that we have screened with the maize *wx* locus assay. Each field-grade formulation of the pesticide was applied to a 10 m x 3 m subplot at the South Farms of the University of Illinois, and inbred W22 kernels homoallelic for *wx-C* were planted. The plants were allowed to grow to early anthesis, at which time the tassels were harvested, stored, and analyzed. The analysis of the pollen grains for reversion to the dominant allele (*Wx*) is based on the fact that a pollen grain carrying the *wx-C* allele does not synthesize the carbohydrate amylose (36, 38). The *wx* revertants are detected by an iodine stain technique (44). When a *wx* heteroallele reverts to *Wx*, amylose is synthesized and incorporated into the starch of the pollen grain. Iodine combines with amylose and forms a blue-black complex (52). Pollen grains that stain blue-black are scored as revertants.

The data presented in Table 2 were collected by the following procedure. Each tassel was removed from its storage jar and agitated in clean 70% ethanol to remove any contaminant field corn pollen grains from the surface of the tassel. Approximately 15 unopened florets were removed from the tassel and agitated in a petri dish filled with 70% ethanol. The anthers were dissected from unopened florets and placed in a stainless steel cup of a VirTis microhomogenizer containing 0.6 ml of a gelatin-iodine stain. The anthers were minced with scissors and homogenized for 30 sec. The homogenate was strained through cheesecloth onto the surface of a large microscope slide, and a coverslip was placed upon the pollen suspension. An additional 0.4 ml of stain was placed into the stainless steel cup and the anthers were rehomogenized for 20 sec. The suspension was then used to make a second slide. After the pollen suspension solidified the slide was examined under a dissecting microscope. The number of blue-black staining pollen grains on the slide were counted. The total number of pollen grains per slide was estimated by counting the number of pollen grains within 20 randomly chosen 1 mm² areas and multiplying this value by an appropriate factor. After a number of slides were analyzed, the *wx* reversion frequency was calculated for each plant by dividing the total number of *Wx* pollen grains by the total estimated number of pollen grains analyzed. After the *wx* reversion frequency of all the surviving

plants of a subplot was determined the mean and the standard error of the mean for each treatment group was calculated.

The data indicate that the *s*-triazine herbicides atrazine, simazine, and cyanazine induce point mutations at the *wx* locus of maize. These data are consistent with the findings on mutation induction in barley (8) and *Drosophila* (29-31). These data also agree with the results of Rogers and Warren that atrazine and cyanazine are activated by maize into a mutagen and with the *in vivo* and *in vitro* plant activation studies of Gentile and Plewa (2, 3) (Table 1). However, the *s*-triazine, procyazine, does not appear to increase the *wx* reversion frequency in maize. Unfortunately this compound has not been analyzed as extensively as the other *s*-triazine herbicides discussed above. For comparison, the data obtained with the insecticides chlordane and heptachlor are included in Table 2. Both of these insecticides increase the reversion frequency of *wx*, however, when these two pesticides were analyzed for *in vivo* plant activation a negative response was observed (Table 1). The herbicides eradican and metolachlor are not mutagenic in the *wx* assay. Combinations of these herbicides with *s*-triazines show an increased reversion frequency. It is not yet resolved whether the increased reversion frequency of these herbicide combinations are due solely to the *s*-triazine component or to a synergistic effect. Additional experience with this assay is required before an understanding of its mutagenic resolving power under *in situ* conditions can be evaluated.

NOTE ADDED IN PROOF: The herbicide simazine is registered as a neoplastic agent, and the herbicide atrazine and cyanazine are included in *Suspected Carcinogens* (53). Sodium azide has recently been reported to be activated by barley into a mutagenic metabolite (54).

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